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# **Elevated CD54 expression renders CD4<sup>+</sup> T cells susceptible to NK cell-mediated killing**

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**Summary:** In this study, a high CD54 expression on CD4<sup>+</sup> T cells was found in HIV-infected individuals, and CD54 expression leads to the killing of CD4<sup>+</sup> T cells by NK cells. Metformin can suppress CD54 expression by inhibiting NF- $\kappa$ B/p65 phosphorylation.

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## Abstract

A decreased number of CD4<sup>+</sup> T cells is a major feature of HIV infection. Here we detected high CD54 expression on CD4<sup>+</sup> T cells in HIV-infected individuals, and demonstrate that upregulated CD54 is associated with disease progression in individuals infected with HIV. We also show that CD54 expression leads to the killing of CD4<sup>+</sup> T cells by NK cells in vitro, and that this is modulated by NF-κB/p65 signaling. Further, we demonstrate that metformin can suppress CD54 expression on CD4<sup>+</sup> T cells by inhibiting NF-κB/p65 phosphorylation; thus, our data suggest that further studies to evaluate the potential role of metformin as adjunctive therapy to reconstitute immune function in HIV-infected individuals are warranted.

**Key words:** CD4<sup>+</sup> T cells; CD54; HIV; NK cell-mediated killing

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## Introduction

Natural killer (NK) cells are an important type of effector cell in the innate immune response, and they exert anti-viral and anti-tumor effects, mainly through cytotoxicity and cytokine secretion[1]. NK cells also have a role in regulation to influence immune responses for the stimulation or inhibition of T cell functions[2]. Recent studies have demonstrated that NK cells can negatively regulate T cell responses directly, by recognizing and killing T cells during lymphocytic choriomeningitis virus[3], Epstein–Barr virus[4], or hepatitis B virus infection[5].

Acquired immunodeficiency syndrome is a serious global infectious disease caused by the human immunodeficiency virus (HIV), and a decrease in CD4<sup>+</sup> T cell counts is the most important feature of HIV infection[6]. Several studies have indicated that NK cells may influence T cells during HIV infection. Vieillard et al. conducted in vitro experiments demonstrating that activated NK cells can directly kill CD4<sup>+</sup> T cells expressing NKp44L in HIV-infected individuals[7], while expression of NKp44L leads to the depletion of uninfected CD4<sup>+</sup> T cells by activated NK cells after simian HIV infection[8]. Richard et al. also found that the HIV-1 Vpr protein significantly up-regulates the expression of ULBP-2, an NKG2D ligand, on CD4<sup>+</sup> T cells, and that CD4<sup>+</sup> T cells expressing this ligand are highly susceptible to killing by unactivated NK cells[9]. Numerous other ligands of NK cell receptors are expressed on CD4<sup>+</sup> T

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cells, and whether they also influence the regulation of interactions between NK cells and CD4<sup>+</sup> T cells requires further study.

In this study, using RNA sequencing (RNA-Seq) analysis, we found that levels of CD54 (also termed intercellular adhesion molecule 1, ICAM-1) were significantly elevated after HIV infection, and that CD54 expression was negatively correlated with CD4<sup>+</sup> T-cell counts in HIV-infected individuals. In vitro experiments revealed that expression of CD54 could mediate the killing of CD4<sup>+</sup> T cells by NK cells. Further, we found that increased CD54 expression can be induced by phosphorylation of nuclear factor- $\kappa$ B (NF- $\kappa$ B/p65), while metformin can reduce the killing of CD4<sup>+</sup> T cells, by down-regulating CD54 expression. These results perhaps suggest that further studies to evaluate the potential role of metformin as adjunctive therapy to reconstitute immune function in HIV-infected individuals are warranted.

## **Methods**

### **Study participants**

In total, 51 HIV-infected individuals and 15 healthy controls participated in this study. The demographic information and clinical characteristics of subjects enrolled in this study are listed in Table 1, including estimated infection days, CD4<sup>+</sup> T cell count and viral load. The method of estimating the length of time of HIV infection has been described previously[10]. Briefly, the estimated time of HIV infection was determined

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primarily by a combination of the clear recall of the patient of the time of high-risk exposure and Fiebig stage. Fiebig stage refers to screening tests (HIV combi, Roche), diagnosis tests (HIV 1+2 Antibody Detection Kit, MP Biomedical Asia Pacific Private Ltd., Singapore) and nucleic acid amplification testing (Roche Molecular Systems, USA) for HIV infection. All HIV-infected individuals were enrolled from a cohort of Red Ribbon clinics in the First Affiliated Hospital of China Medical University (Shenyang, China), which included 15 ART-naïve HIV-infected individuals, 17 immunological responders (IR, whose CD4<sup>+</sup> T cell counts > 500 cells/ $\mu$ l) and 19 Immunological non responders (INR, whose CD4<sup>+</sup> T cell counts < 350 cells/ $\mu$ l)[11]. In our study, IR and INR were selected for the convenience in the clinic, according to CD4<sup>+</sup> T cell counts after two-years of ART.

The study was approved by the Research and Ethics Committee of the First Affiliated Hospital of China Medical University according to the principles of the Declaration of Helsinki, and each subject provided written informed consent for participation.

### **NK cell-mediated killing of CD4<sup>+</sup> T cells**

Primary CD4<sup>+</sup> T (purity > 98.5%) and NK (purity > 97.5%) cells were purified from HIV-infected individuals and controls using immunomagnetic negative selection kits (Stemcell). NK cells were stimulated using IL-12 (10 ng/mL, R&D), IL-15 (50 ng/mL, R&D), and IL-18 (100 ng/mL, R&D) in Roswell Park Memorial Institute

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(RPMI)-1640 (Hyclone) culture medium, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin for 2 days; simultaneously, CD4<sup>+</sup> T cells from controls were cultured with or without stimulation using anti-CD3/CD28 for 2 days (2.5 µg/mL, BD Biosciences). CD4<sup>+</sup> T cells were then co-cultured with autologous NK cells at an E:T cell ratio of 0:1, 0.5:1, 1:1, 5:1, or 10:1, in 96-well U-bottomed plates with 5% CO<sub>2</sub> at 37°C for 5 h. Cells were washed, and surface-stained with CD4-APC-Cy7 and 7-AAD for flow cytometry assays.

#### **Infection with the HIV-1 NL4-3 strain in vitro**

Purified primary CD4<sup>+</sup> T cells were stimulated with phytohemagglutinin (5 µg/mL, Sigma) and IL-2 (20 U/mL, Roche) for 1 day, and infected with the HIV-1 NL4-3 strain for 2 h at 37°C in a total volume of 500 µL. Cells were then washed and cultured in 1 ml of complete RPMI 1640. After 2 days, cells were washed and stained for flow cytometry assays.

#### **Assay of the effect of CD54 blocking on NK cell-mediated killing**

Primary CD4<sup>+</sup> T and NK cells were purified from healthy controls. CD4<sup>+</sup> T cells were stimulated with anti-CD3/CD28, and NK cells were stimulated with IL-12, IL-15, and IL-18 for 2 days. CD4<sup>+</sup> T cells were pre-treated with anti-CD54-blocking Abs or isotype control Abs (Biolegend) for 1 h at 37°C, then co-cultured with autologous NK

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cells at an E:T ratio of 5:1 for 5 h. Cells were then washed and surface-stained for flow cytometry.

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### **Detection of the effects of PDTC**

Purified primary CD4<sup>+</sup> T cells from controls were pre-treated with the NF-κB inhibitor, PDTC (100 μM, R&D), for 18 h, and then CD4<sup>+</sup> T cells were stimulated with anti-CD3/CD28 for 1 h. Cells were analyzed by flow cytometry and real-time quantitative PCR (RT-qPCR).

### **Detection of the influence of metformin on CD54 expression**

Purified primary CD4<sup>+</sup> T cells from controls were pre-treated with metformin/1,1-dimethylbiguanide hydrochloride (Sigma-Aldrich), at various concentrations for 2 h. Then, CD4<sup>+</sup> T cells were stimulated with anti-CD3/CD28 for 24 h, followed by washing and surface-staining for flow cytometry.

### **siRNA transfection**

siRNAs and non-specific Stealth RNAi<sup>®</sup> Negative Control Duplexes were purchased from Thermo Fisher Scientific (Waltham, USA). Purified primary CD4<sup>+</sup> T cells and NK cells were transfected with siRNA using Lipofectamine<sup>®</sup> RNAiMAX reagent (Invitrogen), according to the manufacturer's protocol. Briefly, 10 μM siRNA were mixed with RNAiMAX and added to cells that were 60%–80% confluent. After 48 h, cells were analyzed by flow cytometry and RT-qPCR.

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## Polychromatic flow cytometry

CD4<sup>+</sup> T cells from freshly isolated PBMCs were analyzed using an LSR II Fortessa cytometer (BD Biosciences) calibrated for use with UltraComp eBeads™ Compensation Beads (Invitrogen). Four separate staining panels were used: 1) CD54 expression: CD3-PerCP-Cy5.5, CD4-APC-Cy7, CD54-PE, and PE mouse IgG1  $\kappa$  isotype control (BD Biosciences); 2) Intracellular P24 assay: CD3-PerCP-Cy5.5, CD4-APC-Cy7, CD54-PE, PE mouse IgG1  $\kappa$  isotype control (BD Biosciences), and anti-p24-FITC (Beckman); 3) NK cell specific lysis assay: CD3-PerCP-Cy5.5, CD4-APC-Cy7, and 7-AAD (BD Biosciences); and 4) P65 Phosphorylation assay: CD3-PerCP-Cy5.5, CD4-APC-Cy7, CD54-PE, Phospho-NF $\kappa$ B p65 Monoclonal Antibody (PE), and Mouse IgG2a kappa Isotype Control (PE) (eBiosciences). Violet LIVE/DEAD™ Fixable Dead Cell Stain Kits (Invitrogen) were used to evaluate cell viability. Single cells were gated according to their forward scatter height (FSC-H) and forward scatter area (FSC-A), and Violet LIVE/DEAD negative cells were classified as live cells. Briefly, freshly isolated cells were washed and stained at 4°C with the corresponding Ab cocktail for 30 min. Samples labeled with 7-AAD were analyzed within 15 min; therefore, stained cells were washed and resuspended again with PBS, and analyzed immediately. Samples for intracellular P24 assay were fixed and permeabilized using the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences), and samples for p65 phosphorylation assay were fixed and

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permeabilized using the Intracellular Fixation & Permeabilization Buffer (eBiosciences), according to the manufacturer's recommendations. Samples were acquired on an LSR II Fortessa cytometer (BD Biosciences), and data analysis was performed using FSCDiva and FlowJo software (Treestar).

### **Reverse transcription and quantitative real-time PCR**

Total RNA was isolated from CD4<sup>+</sup> T cells using the RNeasy Micro Kit (Qiagen, Germany) and reverse transcribed using a PrimeScript<sup>®</sup> RT reagent kit (TAKARA, Japan), following the manufacturer's protocol. Real-time PCR for the detection of mRNA was performed using SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (TAKARA). All primers were synthesized by BGI (Beijing, China), and their sequences are listed in Supplemental Table 1. mRNA expression levels were normalized to those of GAPDH. Changes in mRNA expression were calculated according to the  $2^{-\Delta\Delta C_t}$  method[12].

### **Statistical analysis**

The non-parametric Mann-Whitney U test and non-parametric Wilcoxon matched-pairs signed-rank test were used for comparisons of data between two groups. Associations between two groups were evaluated using the Spearman's rank test. A p-value < 0.05 (two-tailed) was considered statistically significant. All data analysis was performed using SPSS 20.0 (IBM, USA) and Prism Version 6.0 (GraphPad Software, USA) software.

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## Results

### High CD54 expression on CD4<sup>+</sup> T cells in HIV-infected individuals

Our team conducted RNA-Seq using peripheral blood mononuclear cell (PBMC) samples from three anti-retroviral therapy (ART)-naïve HIV-infected individuals and three controls, as previously described[13]. In this study, we conducted further data analysis, and found that the level of CD54 expression was much higher in HIV-infected individuals, while those of ULBP-2 and NKP44L were not significantly increased (Figure 1A, B). Subsequently, isolated CD4<sup>+</sup> T cells were used for validation experiments, and the results also demonstrated significantly up-regulated CD54 mRNA levels in ART-naïve HIV-infected individuals compared with controls, while there were no differences in the levels of ULBP-2 and NKP44L (Figure 1C). CD54 expression was further detected by flow cytometry, and the results demonstrated that all ART-naïve HIV-infected individuals had considerably higher CD54 expression on CD4<sup>+</sup> T cells compared with controls, as shown by representative flow cytometry plots (Figure 1D) and statistical analysis (Figure 1E). Moreover, we found that CD54 expression was higher in INR compared with IR and controls (Figure 1F). As expected, CD54 expression was negatively correlated with CD4<sup>+</sup> T-cell counts in all HIV-infected individuals (Figure 1G).

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### **CD54 expression is elevated on CD4<sup>+</sup> T cells after in vitro activation**

It is established that the majority of CD4<sup>+</sup> T cells undergoing death during HIV infection are not infected[14]. To explore whether CD54 expression was also up-regulated in uninfected CD4<sup>+</sup> T cells, we performed infection experiments using isolated CD4<sup>+</sup> T cells and the HIV-1 NL4-3 strain in vitro, and initially observed that CD54 expression was elevated on both infected (p24<sup>+</sup> CD4<sup>+</sup> T cells) and uninfected (p24<sup>-</sup> CD4<sup>+</sup> T cells) CD4<sup>+</sup> T cells (Figure 2A, B). As uninfected CD4<sup>+</sup> T cells exhibit highly significant immune activation after HIV infection[15], purified primary CD4<sup>+</sup> T cells were stimulated with anti-CD3/CD28 in vitro, and the resulting data showed that both the percentage (Figure 2C) and mean fluorescence intensity (MFI) (Figure 2D) of CD54 on CD4<sup>+</sup> T cells were up-regulated by this activation.

### **CD4<sup>+</sup> T cells with CD54 expression are susceptible to NK cell-mediated killing**

NK cells can exert negative immunomodulatory functions by killing autologous T cells[16]. To determine whether NK cells exhibited cytotoxicity against CD4<sup>+</sup> T cells, we sorted autologous NK cells and co-cultured them with CD4<sup>+</sup> T cells from HIV-infected individuals or controls (Figure 3A). Flow cytometry and data analysis indicated that CD4<sup>+</sup> T cells from controls could not be killed by autologous NK cells, whereas CD4<sup>+</sup> T cells from HIV-infected individuals could, and the killing effect was

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stronger when effector to target (E:T) cell ratios were increased (Figure 3B, C). Since HIV infection can lead to activation of CD4<sup>+</sup> T cells in vivo[17], we next stimulated CD4<sup>+</sup> T cells from controls with anti-CD3/CD28 in vitro, and found that activated CD4<sup>+</sup> T cells were also killed by autologous NK cells.

Our results demonstrate that CD54 expression is elevated on uninfected, but activated, CD4<sup>+</sup> T cells, and that activation of CD4<sup>+</sup> T cells leads to increased NK cell-mediated killing; therefore, we further investigated whether CD54 expression was involved in NK cell-mediated killing using experiments blocking or knocking down CD54. A lower proportion of CD4<sup>+</sup> T cells pre-treated with anti-CD54 blocking antibodies (Abs) were killed by NK cells compared with controls, and higher concentrations of anti-CD54 blocking Ab led to lower cytotoxicity against activated CD4<sup>+</sup> T cells (Figure 4A, B). To further validate the results obtained using blocking Abs, siRNA targeting CD54 was transfected into CD4<sup>+</sup> T cells to inhibit CD54 expression, and could successfully reduce CD54 mRNA levels (Figure 4C) as well significantly decreasing CD54 protein expression on CD4<sup>+</sup> T cells (Figure 4D). As illustrated in Figure 4E and Figure 4F, CD4<sup>+</sup> T cells with CD54 knocked down were less frequently killed by NK cells compared with controls. LFA-1, a receptor on NK cells, has been widely reported as a CD54 ligand, involved in NK cell cytotoxicity[18]. Next, we transfected siRNA targeting LFA-1 into NK cells to inhibit LFA-1 expression (Figure 4G). We found that knock-down of LFA-1 in NK cells could also

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reduce killing of CD4<sup>+</sup> T cells by NK cells (Figure 4H). Taken together, our results suggest that CD54 expression on CD4<sup>+</sup> T cells induces NK cell cytotoxicity, which may contribute to the killing of CD4<sup>+</sup> T cells during HIV infection.

### **CD54 expression on CD4<sup>+</sup> T cells is modulated by NF-κB/p65**

Based on our observations regarding the role of CD54 on CD4<sup>+</sup> T cells, we further investigated potential mechanisms affecting CD54 expression. The important signaling molecule, NF-κB/p65, has been previously reported to up-regulate CD54 expression in various endothelial cells[19]; therefore, we determined whether NF-κB/p65 had a similar effect in CD4<sup>+</sup> T cells. Our data showed that NF-κB/p65 phosphorylation was increased in CD4<sup>+</sup> T cells following activation (Figure 5A, B), and the proportion of NF-κB/p65 phosphorylation was positively correlated with CD54 expression on CD4<sup>+</sup> T cells (Figure 5C). Subsequently, we transfected CD4<sup>+</sup> T cells with siRNA targeting NF-κB/p65 (si-NF-κB/p65) or control siRNA, and found that it led to a marked depletion of NF-κB/p65 mRNA levels (Figure 5D). Further, we observed that knockdown of NF-κB/p65 significantly down-regulated expression of CD54 mRNA (Figure 5E) and protein on the cell surface (Figure 5F). Subsequently, to verify these results, we used the NF-κB inhibitor, pyrrolidinedithiocarbamate (PDTC)[20, 21], to suppress NF-κB/p65 phosphorylation (Figure 5G), and found that CD54 expression was markedly inhibited by PDTC (Figure 5H). Our data suggest that transcription and protein levels of CD54 in CD4<sup>+</sup> T cells can be promoted by

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NF- $\kappa$ B/p65 phosphorylation; hence, inhibition of NF- $\kappa$ B/p65 phosphorylation contributes to reduced expression of CD54 on CD4<sup>+</sup> T cells.

### **Metformin reduces CD54 expression by inhibiting NF- $\kappa$ B/p65 phosphorylation**

Our results demonstrate that NF- $\kappa$ B/p65 phosphorylation in CD4<sup>+</sup> T cells promotes CD54 expression, resulting in the killing of CD4<sup>+</sup> T cells by NK cells. Thus, we should find ways to reduce CD54 expression and avoid the decrease in uninfected CD4<sup>+</sup> T cells during HIV infection. Although our experiments demonstrate that PDTC (an NF- $\kappa$ B/p65 inhibitor) can reduce CD54 expression, it is not suitable for use as a clinical intervention because of its toxicity[22]. Metformin, an anti-type II diabetes agent, has been reported to have a clear inhibitory effect on NF- $\kappa$ B/p65[23, 24]. Therefore, we incubated metformin with activated CD4<sup>+</sup> T cells and evaluated levels of NF- $\kappa$ B/p65 phosphorylation and CD54 expression. We observed that phosphorylation of NF- $\kappa$ B/p65 in CD4<sup>+</sup> T cells was suppressed by metformin (Figure 6A), as was CD54 expression (Figure 6B, C). Further, higher concentrations of metformin reduced CD54 expression more strongly (Figure 6D). Subsequently, we co-cultured metformin, CD4<sup>+</sup> T cells, and autologous NK cells, and found that metformin could effectively inhibit the killing of CD4<sup>+</sup> T cells by NK cells (Figure 6E, 6F). Taken together, our results suggested that metformin could be used as a clinical intervention to inhibit the killing of CD4<sup>+</sup> T cells by autologous NK cells, and assist in immune reconstitution in HIV-infected individuals.

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## Discussion

The CD54 molecule is a member of the immunoglobulin superfamily of adhesion molecules, which are expressed on the surface of various cells, including leukocytes, endothelial cells, and smooth muscle cells[25]. CD54 contributes to the directed migration of leukocytes across endothelial cell layers to sites of inflammation, as well as being involved in cell-to-cell interactions[26], including during the transmission of HIV[27]. Several studies have confirmed that CD54 and soluble CD54 (sCD54) are significantly elevated in response to inflammatory diseases, injuries, and tumors[28, 29].

Our results demonstrate that CD54 expression is considerably elevated on CD4<sup>+</sup> T cells from ART-naïve HIV-infected individuals, as previously reported[30]. For the first time, we found that CD54 expression was much higher in INR after two-years of ART and was significantly associated with disease progression. More importantly, we demonstrated that CD54 expression is remarkably increased on HIV-infected (P24-positive) CD4<sup>+</sup> T cells and also on uninfected (P24-negative) CD4<sup>+</sup> T cells in an *in vitro* culture system for HIV-1 NL4-3 infection. It has been reported that HIV-1 Tat can promote CD54 expression via Akt signaling[31], while another report demonstrated that HIV Vpu down-modulates CD54 expression[32]. In our study, we found that the entire live virus of HIV (HIV-1 NL4-3) could up-regulate CD54 expression, which includes various components of the virus and might resist the effect

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of Vpu protein. It has been reported that there is still a high level of chronic activation in INR, despite the low levels of HIV RNA[33]. Our data also demonstrate that samples from INR had higher CD54 expression levels on CD4<sup>+</sup> T cells, indicating that activation of T cells has an important role in promoting CD54 expression. Meanwhile, our study found that both HIV infection and TCR activation can promote CD54 expression. HIV infection contributes to immune activation[34], while cell activation may also lead to HIV infection[35]. Thus, there is evidence that both HIV infection and T cell activation can contribute to CD54 expression. Moreover, it has been reported that expression levels of ICAM-2 (CD102) and ICAM-3 (CD50) are also elevated after HIV infection[36], and these factors are related to inflammation[37]. Therefore, unraveling further mechanisms on biological significance of ICAM family molecular and HIV pathogenesis could benefit future research.

NK cells can negatively influence immune responses by killing autologous T cells.

Our results demonstrate that elevated CD54 expression induces NK cell-mediated killing of CD4<sup>+</sup> T cells. Killing of CD4<sup>+</sup> T cells by NK cells may be due to the binding of CD54 with LFA-1[38], as we also found that knock-down of LFA-1 on NK cells could reduce the killing of CD4<sup>+</sup> T cells by NK cells. The binding of CD54 to LFA-1 on NK cells promotes the release of IFN- $\gamma$  or cytotoxic granules from NK cells, resulting in cell death, as reported for tumor cells[39, 40]. Moreover, we found

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that the transcription and protein expression of CD54 in CD4<sup>+</sup> T cells was enhanced by NF-κB/p65 phosphorylation. Similarly, it has been reported that the expression of CD54 is regulated by NF-κB/p65 signaling in endothelial cells[19, 41].

Large scale reductions in CD4<sup>+</sup> T cells is a major feature of HIV infection; however, in vivo, only a small proportion of CD4<sup>+</sup> T cells are actually infected with HIV, while the majority are not infected but are activated, and both infected and uninfected cells are killed by immune responses[15, 42]. Although the killing of infected CD4<sup>+</sup> T cells by NK cells can inhibit the replication and transmission of HIV, NK cells also appear to kill a large number of uninfected CD4<sup>+</sup> T cells, leading to exhaustion of the immune response. Therefore, investigation of interventions that can inhibit the killing of CD4<sup>+</sup> T cells by NK cells is strongly warranted. Our results indicated that PDTC could reduce CD54 levels by inhibiting NF-κB/p65 phosphorylation; however, the high toxicity of PDTC[22, 43] limits its clinical application. Furthermore, our results provide evidence supporting the potential use of metformin as a clinical intervention to reduce CD54 expression and avoid the loss of CD4<sup>+</sup> T cells during HIV infection. However, the concentration of metformin currently used for in vitro experiments is higher than that found in the blood of diabetic patients. Thus, the appropriate concentration of metformin for use in future clinical application, aimed at immunological reconstitution, requires further study. Metformin is not only a therapeutic drug for diabetes, but is also reported to significantly regulate signaling

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pathways, such as MAPK[44] and mTOR[45]. In support of our results, metformin is also reported to inhibit the activation of NF- $\kappa$ B signaling in epithelial cells[46] and cancer cells[47]. Other studies have also reported that levels of CD54 can be modulated by the MAPK-p38[48], FAK/ERK/GSK3, and PKC $\delta$ [49] signaling pathways; however, like NF- $\kappa$ B signaling, the effects of these molecules on CD54 are not specific. Thus, a more targeted approach to blocking CD54 expression would be preferable in future research.

To conclude, we report high CD54 expression on CD4<sup>+</sup> T cells in HIV-infected individuals, association of CD54 expression with disease progression, and upregulation of CD54 in HIV-infected individuals with inferior immune recovery during ART. Meanwhile, our data indicate that CD4<sup>+</sup> T cells expressing CD54 are susceptible to NK cell-mediated killing, and that CD54 expression on CD4<sup>+</sup> T cells is modulated by NF- $\kappa$ B/p65. Furthermore, we prove that metformin can inhibit the killing to CD4<sup>+</sup> T cells by NK cells, perhaps suggesting metformin might be used as the adjunctive therapy for HIV-infected individuals to help restore the number of CD4<sup>+</sup> T cells and reconstitute the immune function in addition to antiretroviral therapy.

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## Author Contributions

XC, HC YJ, and HS conceived the study. XC, ZZ, YF, XH, YZ, JX, HD and HC designed and conducted experiments. XC, XH, YZ, YH and TD recruited participants and analyzed the data. XC, TD YJ, and HS wrote and revised the manuscript. All authors read and approved the final manuscript.

## Competing Interests statement

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Figure legends

**Figure 1.** CD54 expression was increased on CD4<sup>+</sup> T cells in HIV-infected individuals. Relative expression (**A**) and fold of change (**B**) of ULBP-2, NKP44L, and CD54 according to RNA-Seq data from PBMC samples from ART-naïve HIV-infected individuals (n = 3) and controls (n = 3). (**C**) Relative mRNA expression levels of ULBP-2, NKP44L, and CD54 by RT-qPCR in isolated CD4<sup>+</sup> T cells from ART-naïve HIV-infected individuals (n = 5) and controls (n = 5). Representative cytometry dot plots (**D**) and percentages (**E**) of ULBP-2, NKP44L and CD54 on CD4<sup>+</sup>

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T cells from ART-naïve HIV-infected individuals (n = 15) and controls (n = 15). **(F)** Comparison of the percentage of CD54<sup>+</sup> CD4<sup>+</sup> T cells in individuals classified as IR (n = 17) and INR (n = 19), and controls (n = 15). **(G)** Correlation between the percentage of CD54<sup>+</sup> CD4<sup>+</sup> T cells and CD4<sup>+</sup> T cell counts (n = 51). Data were analyzed using the non-parametric Mann-Whitney U test and Spearman's rank test. Error bars indicate median and interquartile range. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

**Figure 2.** CD54 expression was upregulated on CD4<sup>+</sup> T cells after activation in vitro. Representative cytometry dot plots **(A)** and percentages **(B)** of infected (p24<sup>+</sup>) and uninfected (p24<sup>-</sup>) CD4<sup>+</sup> T cells expressing CD54 in an in vitro HIV-1 NL4-3 infection culture system (n = 3). Percentage **(C)** and MFI **(D)** of CD54 expression on CD4<sup>+</sup> T cells stimulated with anti-CD3/CD28 (n = 15). Data were analyzed by non-parametric Mann-Whitney U test and non-parametric Wilcoxon matched-pairs signed-rank test. Error bars indicate median and interquartile range. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

**Figure 3.** NK cell-mediated killing of CD4<sup>+</sup> T cells in HIV-infected individuals and controls. NK cells and autologous CD4<sup>+</sup> T cells from HIV-infected individuals (n = 5) or controls (n = 5) were sorted and co-cultured. NK cells were stimulated with IL-12, IL-15, and IL-18, and CD4<sup>+</sup> T cells from controls were cultured with or without anti-CD3/CD28 stimulation. Representative cytometry dot plots **(A)** and proportions

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(B, C) of 7-AAD<sup>+</sup> CD4<sup>+</sup> T cells after co-culturing with autologous NK cells at effector to target (E:T) cell ratios of 0:1, 0.5:1, 1:1, 5:1, or 10:1. Data were analyzed by non-parametric Mann-Whitney U test. Error bars indicate median and interquartile range. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001.

**Figure 4.** CD4<sup>+</sup> T cells expressing CD54 were susceptible to NK cell-mediated killing. CD4<sup>+</sup> T cells were stimulated with anti-CD3/CD28; simultaneously, NK cells were stimulated with IL-12/IL-15/IL-18 for 2 days. CD4<sup>+</sup> T cells were pre-treated with anti-CD54-blocking Abs or isotype control Abs for 1 h and co-cultured with autologous NK cells. Representative cytometry dot plots (A) and proportions (B) of CD4<sup>+</sup> T cells killed by NK cells in controls samples (n = 4) treated with different concentrations of anti-CD54 blocking Ab or isotype control Ab. Relative mRNA (C) and protein (D) levels of CD54 on activated CD4<sup>+</sup> T cells (n = 3) transfected with CD54 siRNA. Representative cytometry dot plots (E) and proportions (F) of NK cell-mediated killing of activated CD4<sup>+</sup> T cells (n = 3) pre-transfected with CD54 siRNA. (G) Relative mRNA expression levels of LFA-1 in NK cells transfected with LFA-1 siRNA. (H) NK cell-mediated killing of activated CD4<sup>+</sup> T cells (n = 3) pre-transfected with LFA-1 siRNA in NK cells. Data were analyzed by non-parametric Mann-Whitney U test. Error bars indicate median and interquartile range. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001.

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**Figure 5.** CD54 expression on CD4<sup>+</sup> T cells was modulated by NF-κB/p65.

Representative flow charts (**A**) and percentages (**B**) of NF-κB/p65 phosphorylation in activated CD4<sup>+</sup> T cells (n = 11). (**C**) Correlation between the percentage of NF-κB/p65 phosphorylation and CD54 expression on activated CD4<sup>+</sup> T cells (n = 11). (**D**) Relative expression of NF-κB/p65 mRNA in activated CD4<sup>+</sup> T cells after transfection with NF-κB/p65 siRNA (n = 3). Relative mRNA (**E**) and protein (**F**) levels of CD54 on activated CD4<sup>+</sup> T cells after transfection with NF-κB/p65 siRNA (n = 3). The proportion of NF-κB/p65 phosphorylation (**G**) and CD54 expression (**H**) on activated CD4<sup>+</sup> T cells pre-treated with PDTTC (n = 11). Data were analyzed by non-parametric Wilcoxon matched-pairs signed-rank test and Spearman's rank test. Error bars indicate median and interquartile range. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

**Figure 6.** Metformin reduced CD54 expression by inhibiting NF-κB/p65 phosphorylation. (**A**) Percentage of NF-κB/p65 phosphorylation in activated CD4<sup>+</sup> T cells pre-treated with metformin (n = 6). Representative cytometry dot plots (**B**) and percentages (**C**) of CD54 expression on activated CD4<sup>+</sup> T cells pre-treated with metformin (n = 6). (**D**) Percentage of CD54 expression on activated CD4<sup>+</sup> T cells pre-treated with metformin at different concentrations (n = 6). Representative flow charts (**E**) and proportions (**F**) of NK cell-mediated killing of CD4<sup>+</sup> T cells treated with PDTTC (50 μmol/L) or metformin (16 μg/mL) (n = 3). Data were analyzed by

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non-parametric Mann-Whitney U test and non-parametric Wilcoxon matched-pairs signed-rank test. Error bars indicate median and interquartile range. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

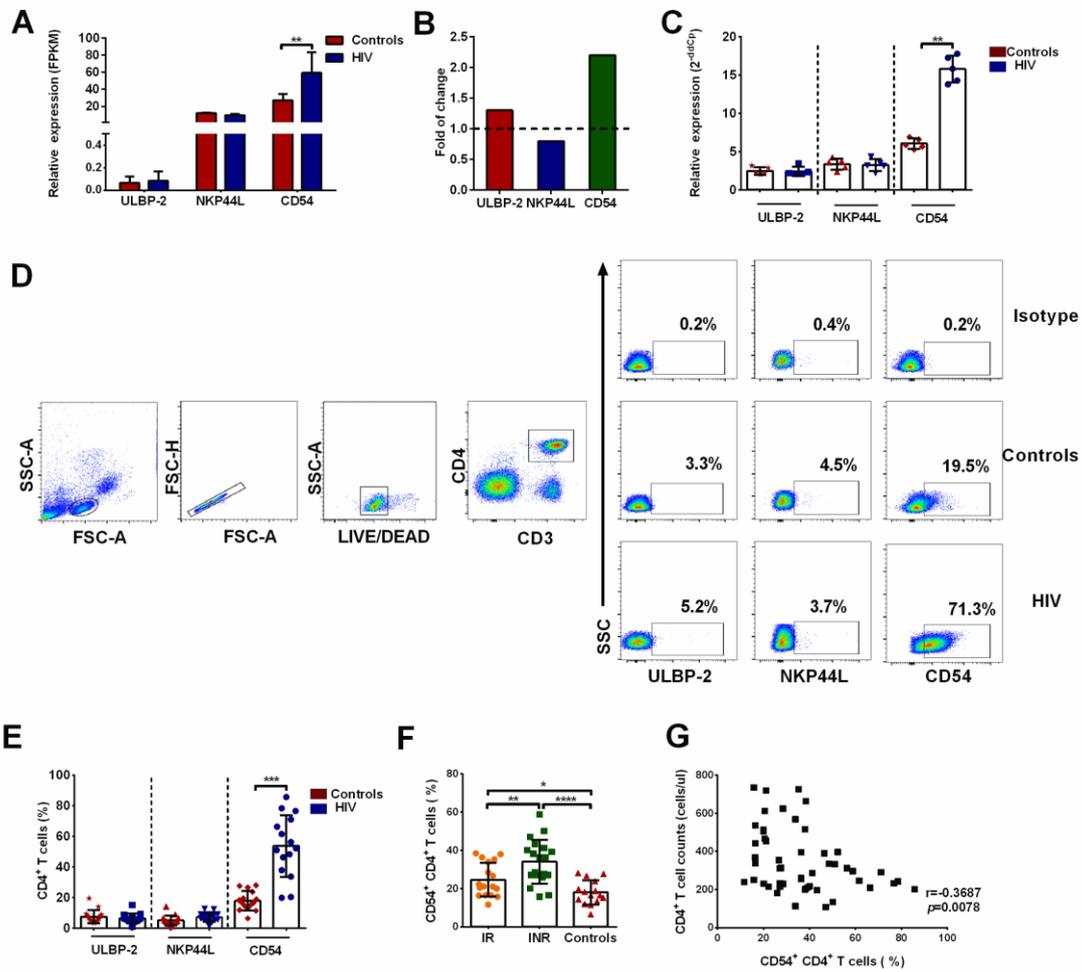
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**Table 1 Clinical characteristics of subjects enrolled in this study**

Characteristics	ART-naïve HIV individuals	Two-year ART HIV individuals		Healthy controls
		IR	INR	
Subject no.	15	17	19	15
Age (years); median (IQR)	32 (27, 40)	31 (26, 41)	30 (24, 39)	29 (23, 42)
Male (No, %)	15 (100%)	17 (100%)	19 (100%)	15 (100%)
Estimated infection days; median (IQR)	74 (52, 115)	887 (765,1011)	901 (773, 1124)	N/A
CD4 <sup>+</sup> T cell count (cells/μl); median (IQR)	372 (206, 433)	566 (509, 668)	273 (198, 302)	877 (732, 983)
Viral load (copies/ml); median (IQR)	81850 (11250, 708350)	< 20	< 20	N/A

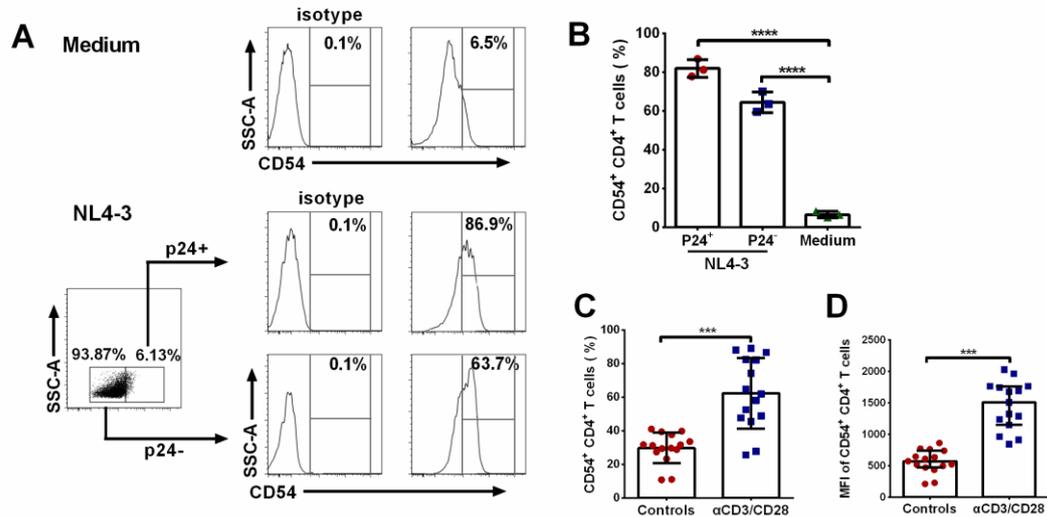
ART, anti-retroviral therapy; IR, immunological responders; INR, immunological non responders; IQR, interquartile range

**Figure 1**



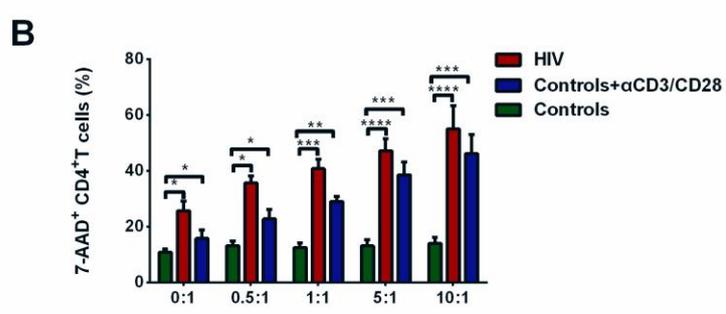
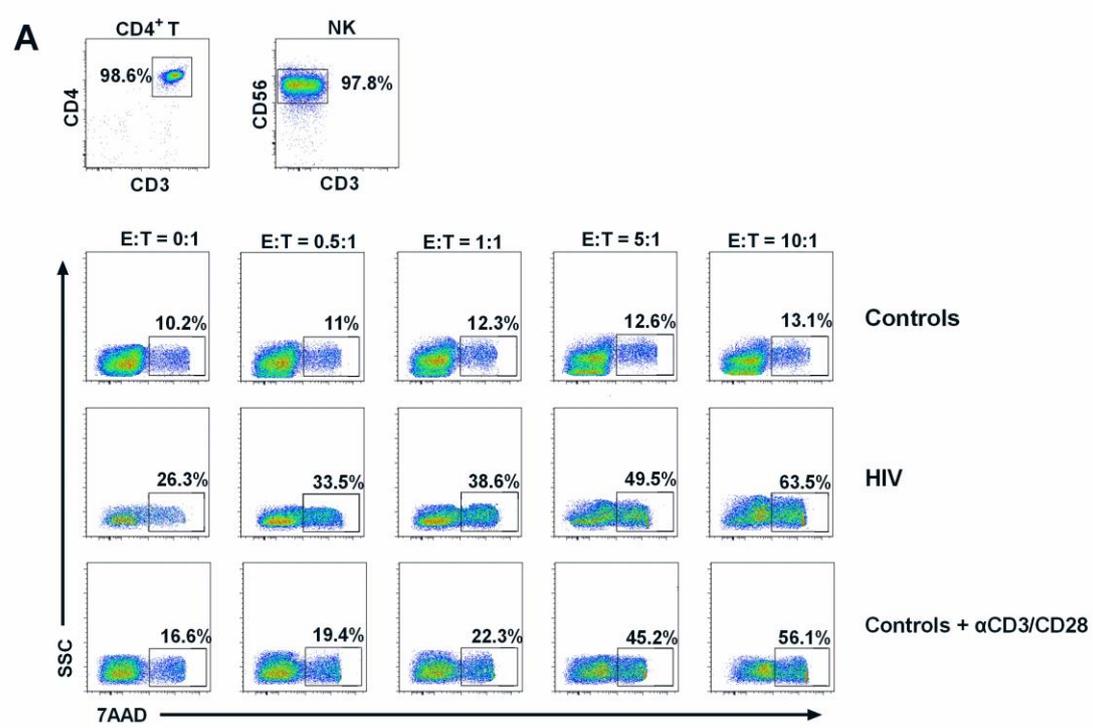
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Figure 2



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**Figure 3**



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**Figure 4**

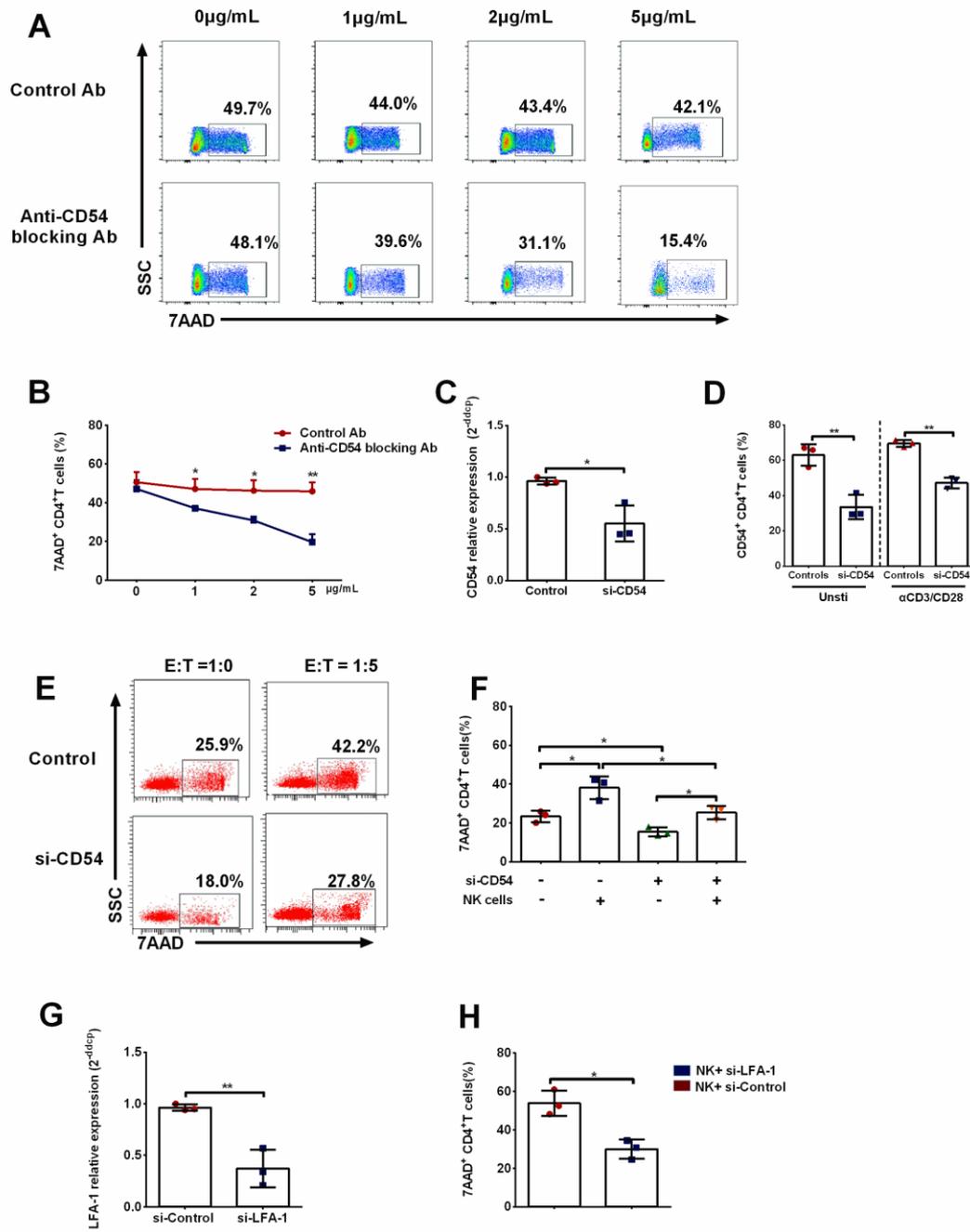
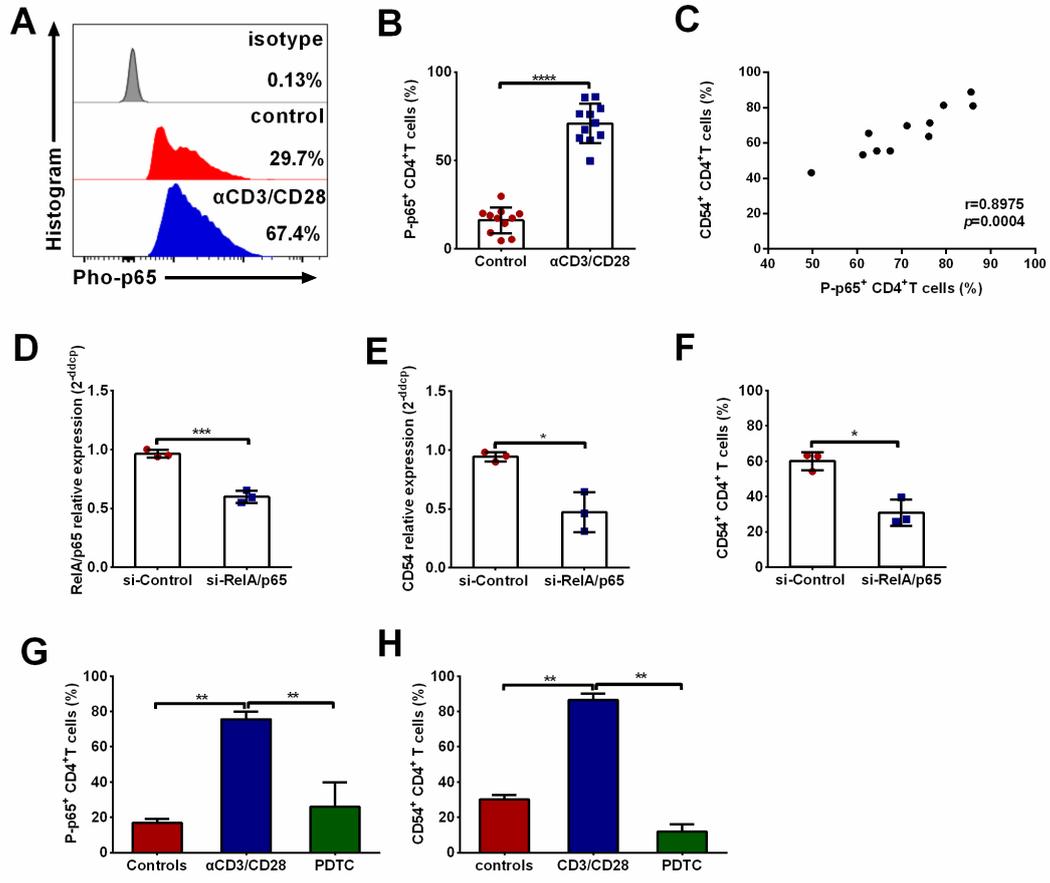


Figure 5



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**Figure 6**

